IDENTIFICATION OF ALCOHOL-SOLUBLE HEMOLYSINS IN BLOOD SERUM.*

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IN a preliminary communication[1] it has been reported that ethyl-alcohol extracts from blood serum of various animals contain a substance or substances which lake red blood corpuscles held in suspension in 0.8 per cent. NaCl solution.

These hemolytically active extracts may be obtained from fresh serum, from dialysed serum, indeed from fresh serum which has been heated to any temperature up to 100° C. The lysin is therefore chemically quite stable; it is not generated by decomposition processes which may occur in blood serum, nor is it a product of the enzymatic process which Hahn[2] has recently shown occurs in blood of normal animals after withdrawal from the animal body; and its formation does not depend—wholly at least—(as may be inferred more fully from what will be presented farther on) on the action of the lipase of blood serum described by Hanriot.[3]

I have usually prepared it from serum, which, after being mixed with a few drops of chloroform, has been dried slowly in an incubator. The bone-dry serum is pulverized, then brought into a Soxhlet extraction apparatus and digested for several hours with petroleum ether. After this treatment the serum is digested with ethyl alcohol, preferably 60–75 per cent. Absolute alcohol removes some of the lysin also, but not to the extent that alcohol containing water does, which obviously penetrates the vitreous dried serum better than the dry absolute alcohol. The digestion fluid is filtered into wide, flat dishes and allowed to evaporate slowly in an incubator to complete dryness. The presence of

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[1] *Trans. of the Chicago Path. Soc.*, 1904, 6, p. 128.

[2] *Münch. med. Wchnschr.*, No. 16, 1904, 51, p. 689.

[3] *Comp. rend. heb. des séances de l'académie des sciences*, 1896, 123, p. 753; 1897, 124, pp. 235, 778.

the hemolysin in such extracts does not depend entirely—if at all—on any alteration which may occur in the serum (lipase) or in the extract (influence of light) during the drying in the incubator, because mixing fresh blood serum with two parts of 96 per cent. alcohol, drawing off the supernatant fluid after centrifuging the mixture, and evaporating it in a vacuum removed from sunlight also yields a hemolytic extract. Such extracts are, however, not as active hemolytically as those obtained from dried serum and dried in an incubator. It is possible, therefore, that even the slow dryings in the incubator are accompanied by some changes in the serum and in the composition of the extract which intensifies its hemolytic properties; but the effect of drying in the incubator is by no means essential to the formation of the lytic substance.

The extracts after being freed completely from alcohol are mixed with sterilized 0.8 per cent. NaCl solution of a volume equal to the volume of serum used at the outset. This mixture furnishes a permanent emulsion which is neutral to litmus.

The emulsions lake red blood corpuscles of any animal which has been tested, viz.: sheep, goat, rabbit, ox, hog, guinea pig, dog, and man. Usually about 0.2 c.c. of an emulsion suffice to cause complete laking of one c.c. of a five per cent. suspension of washed corpuscles. The degree of lytic activity of the emulsions varies, however. The minimum amount of an emulsion observed to cause any appreciable hemolysis is 0.02 c.c. Lysis occurs readily at ordinary room temperature, and even at 0° C. The same emulsion acting on corpuscles of different species of animals produces different degrees of hemolysis, sheep corpuscles being apparently the most resistant, and dog corpuscles the most susceptible.

Heating the emulsions, even to 100° C. for three hours, causes no apparent alteration in their hemolytic properties.

The lysin is not in the menstruum, but rather in the suspended matter of the emulsions. For if an emulsion be passed through a Chamberland porcelain filter, the yellow filtrate, if perfectly clear, usually has no lytic action on erythrocytes. If, on the other hand, the residue be dissolved from the filter with alcohol,

the clear solution which is obtained leaves, on being evaporated, a residue which, re-emulsified in salt solution, hemolyses quite as actively as the original emulsion.

The lytic action of these emulsions on the corpuscles of any species of animal is inhibited by the blood serum from the same species. Heated sera from other species may also have antilytic properties, but not of the same degree toward the corpuscles of all species. For instance, laking of dog corpuscles by an active emulsion was not prevented by the same amounts of heated sheep or dog serum as suspended lysis of sheep or guinea pig corpuscles. The watery fluid which exudes from the coagula of boiled serum also has antilytic properties; the same is true of serum which has been dialyzed for forty-two hours and then mixed with an equal volume of 1.6 per cent. NaCl solution.

As for the origin of this lysin: it is not furnished by the white corpuscles, as might be suggested. Alcohol extracts made from white blood corpuscles, obtained from the pleural cavity of a rabbit after injection of aleuronat did not contain it. It was present to a very slight extent in an extract from pleural fluid and also in one from ascites fluid.

The hemolytic properties of these extracts, in so far as similar tests have been made of them, correspond quite closely to those of the organ extracts which Korschun and Morgenroth[1] made by digestions of the organs of animals in physiological salt solution. The probability of the identity of at least one of the active components of the extracts here described and that in their extracts is very strong.

The known constituents of blood serum which are extractable by alcohol but which are not soluble in water and which therefore may be sought in the suspended matter of these emulsions are: fats, cholesterin, cholesterin-esters of fatty acids, lecithin, jecorin fatty acids, and insoluble soaps. Blood serum is said to contain some soluble soap which would of course be extracted by alcohol. The soluble soaps have an intense lytic action on erythrocytes. Since, however, the filtrates obtained by passing the emulsions through the Chamberland filter usually do not lake, it may be

[1] *Berl. klin. Wchnschr.*, 1902, 39, p. 870.

taken for granted that soluble soap is not usually present in the emulsions in sufficient quantity to be a factor in their hemolytic action. The method of extraction used in making the emulsions here described by no means exhausts all the matter extractable by alcohol from blood serum. The amount of extract yielded by boiling fresh serum four or five times with alcohol in a flask fitted with a return condenser is of course much larger than the amounts obtained by the mild procedure actually used. The cold extractions and drying at low temperatures were adhered to in order to obtain the extractable substances more nearly in the state in which they are present in the normal serum. It is known that constituents of blood serum—as jecorin for instance —would certainly be decomposed by a hot extraction by alcohol.

An emulsion of the extract obtained from pulverized dried serum by digesting it for twelve hours with petroleum ether in a Soxhlet extraction apparatus did not lake red corpuscles. This extract would contain fats, probably cholesterin, cholesterin-esters, lecithin, and possibly jecorin. Extracts from dried serum obtained with ethyl ether or with chloroform have hemolytic properties. Such extracts may contain in addition to the substances extracted by petroleum ether fatty acids and insoluble soaps.

To ascertain exactly what the lytic agent is in the alcohol extracts the following procedure was adopted: Pulverized dried serum was digested for twelve hours with petroleum ether in a Soxhlet apparatus. The dried serum was then freed from petroleum ether and treated several times in the course of three days with 60–90% alcohol. The digestion fluid was filtered into a wide, flat dish and allowed to evaporate in an incubator at first, then for two days in a calcium-chloride chamber in the incubator. The now thoroughly dried extract was treated several times with absolute alcohol, the dissolved portion filtered into a dish and again allowed to dry in an incubator and afterwards in the calcium-chloride chamber. The residue of the extract insoluble in absolute alcohol was freed from alcohol and emulsified in salt solution; it was found to have no hemolytic power.

The substance soluble in absolute alcohol, after thorough drying was brought on filter paper into a Soxhlet apparatus and digested at the lowest possible temperature with perfectly water-free petroleum ether. The substance thus dissolved out by petroleum ether, after expulsion of the petroleum ether, was tested for fat and cholesterin with positive results, for lecithin and jecorin with negative results. The substance was then emulsified and found to be non-hemolytic. Thus it was shown that neither fats, cholesterin nor cholesterin-esters are active as hemolytic agents, and since lecithin and jecorin cannot be shown to be present in the extracts they do not come into question as factors in the hemolytic action of the emulsions obtained from serum with weak alcohol.

The portion of the absolute alcohol extract insoluble in petroleum ether was mixed with water, brought into a separating funnel and treated in different cases with chloroform and with ethyl ether. With chloroform a substance was withdrawn from the aqueous mixture, which being dried and emulsified was strongly hemolytic. The aqueous mixture left after the exhaustion with chloroform was evaporated and the resultant residue emulsified and found to be non-hemolytic. With ethyl ether an extract was obtained from the substance left undissolved by petroleum ether which hemolyses and the emulsion of the dried residue was hemolytic also.

Now the substances removable from the aqueous emulsion of the material insoluble in petroleum ether by both chloroform and ethyl ether are the fatty acids. They are undoubtedly the chief factors in the hemolysis caused by these emulsions. Oleic, stearic, and palmitic acids may be emulsified in small quantities in 0.8% NaCl solutions and such emulsions lake red blood corpuscles with surprising facility. The amount of oleic acid held in suspension in 10 c.c. of salt solution is not ordinarily strong enough to neutralize 1.0 c.c. of $\frac{N}{50}$ NaOH solution. Yet one c.c of such an emulsion dissolves the erythrocytes in one c.c. of suspended corpuscles almost immediately and 0.1 c.c. dissolves them almost completely after a few hours. Stearic acid is less active, and palmitic acid is the least active of the three. The

unanalyzed active emulsions derived from blood serum, titrated with $\frac{N}{50}$ NaOH solution, using phenolphthalein as indicator, always show the presence of free fatty acid. The amounts of $\frac{N}{50}$ NaOH solution required to neutralize 20 c.c. of an emulsion being usually 0.8 c.c. to 1.5 c.c. The presence of even that much free fatty acid in an emulsion would, according to tests with prepared emulsions of pure fatty acids in physiological salt solution, almost wholly account for its hemolytic properties. There may be some doubt, however, if even such small amounts of free fatty acids are to be found in blood serum with its normal alkalinity. It is a question if they are not the products of the action of lipase, which by the method usually followed of making the extracts is not to be excluded; furthermore the decomposing effects which light and air are known to have on some fatty compounds might be a source.

Be that as it may, the treatment of an emulsion with ethyl ether which should take up all the fatty acids present does not deprive it wholly of its hemolytic properties. Treatment of dry extracts with chloroform sometimes leaves something in the insoluble residue which in emulsion is hemolytic. Now the substances left by ethyl ether in an emulsion, which one might suspect, would be the soaps. For reasons already set forth it cannot be supposed that any considerable amount of soluble soap is present in the extracts. There remain, then, the insoluble soaps to be considered.

A preparation of magnesium soap made either from ordinary soap or from a solution of pure sodium oleate can be emulsified and held in fine suspension in NaCl solution. Such emulsions, which are perfectly neutral, even to phenolphthalein, have the property of laking red blood corpuscles on being mixed with them. This laking is inhibited by a small quantity of serum; so it cannot be said that the antilytic properties of serum toward these emulsions is to be ascribed to a neutralizing effect which the alkali of the serum may have on free fatty acids. Moreover, this would form soluble soap, which we know is a laking agent. After standing several days the suspended matter of a magnesium soap emulsion gathers together and leaves a clear men-

struum, which on being titrated with silver nitrate solution is shown still to contain just as much chloride of sodium per cubic centimeter as the NaCl solution used. It cannot be said, therefore, that the lytic properties of an emulsion of magnesium soap in NaCl solution are to be ascribed to a hypotonicity of the salt solution brought about by the suspended matter attaching some of the NaCl and thus withdrawing it from the solution.

Calcium soaps prepared in the same way are not so emulsifiable and do not have hemolytic properties.

There is no mention by physiologists that the magnesium and calcium of blood serum are there in combination with fatty acids. Yet, knowing that when blood serum is incinerated, more metallic oxides are found in the ash than can be accounted for by organic acids known to be present, the excess of bases would be explained by the fact that they are in combination with some unknown inorganic acids. Magnesium soap is often found in the intestines, and it is known that by the aid of bile it can readily pass through the intestinal wall, but its fate has never been further traced. The mystery presented by blood serum containing an incompatible mixture such as magnesium and phosphoric acid, calcium and carbonic acid, supposedly all in a solution together, has been explained thus: Perhaps the albumins of a serum have the faculty of holding these substances in solution as water cannot. But the possibility of a combination of calcium and magnesium with fatty acids and held in emulsion in the blood serum removes the mystery. Now, if magnesium soaps are present in the blood serum, they may be included in the substances of the alcohol extracts of blood serum which are hemolytic. A faultless demonstration of the presence of magnesium soaps in the extracts cannot be made so easily, however, on account of the presence of the free fatty acids. A perfect isolation of the fatty acids from the magnesium soaps is very difficult, since all utilizable solvents of the former either partially dissolve or emulsify magnesium soaps. This difficulty is much heightened by the fact that the amounts of these substances in our emulsions are at best very minute. Numerous incinerations of active emulsions have always shown the presence of magnesium and sometimes of

calcium in the ash—in the ash derived from substances exhausted with ethyl ether or chloroform from absolute alcohol extracts, as well as in the residues not taken up by these solvents. There are, one may assert at least, no other known compounds of magnesium in blood serum which are soluble successively in absolute alcohol and chloroform or in absolute alcohol and ether. Hence one is warranted in making the assertion that magnesium soaps are present in blood serum and, that being so, they are factors in the hemolytic action of extracts of blood serum.

This would naturally lead to the inquiry as to whether magnesium soaps, which are evidently present in blood serum in larger quantities than the alcohol extractions from dried serum furnish, may not be concerned in some way with the action of the complex hemolysins of the blood; whether they, for instance, may not be connected in some way with the more stable component of the hemolysins, the amboceptor. To gain a possible clue an endeavor was made, (1) to ascertain if the amounts of calcium and magnesium in the blood serum of an immunized animal showed increase over that of a normal animal; (2) to ascertain if heated immune serum, after being mixed with suitable washed corpuscles, was deprived of appreciable amounts of magnesium or calcium, since the corpuscles absorb the amboceptor. With these considerations in mind 80 c.c. of blood serum from a goat which had been injected periodically with sheep's corpuscles, so that just before the analysis 0.005 c.c. laked completely one c.c. of a five per cent. suspension of washed sheep corpuscles, was heated at 62° C. for one-half hour. The serum was now no longer hemolytic. It was then divided into two equal parts. One part was evaporated to dryness in a large porcelain crucible. The other portion was treated with the washed corpuscles from 100 c.c. of sheep's blood at 36° C. for two hours, then centrifuged and separated from the corpuscles. The corpuscles were then mixed with 0.8 % NaCl solution twice, centrifuged, and separated from the supernatant fluid which was added to the serum. The serum and the corpuscles were then evaporated to dryness separately in porcelain crucibles. Washed corpuscles from 100 c.c. of the same sheep's blood without being treated with goat serum were also

evaporated to dryness in a crucible. Finally 40 c.c. of blood serum from a normal goat was evaporated to dryness. After thorough drying of the contents of these five crucibles they were carefully incinerated.

The ashes resulting from the incineration were dissolved in hydrochloric acid and the resulting solutions analyzed for their calcium and magnesium content. This was done by rendering the solutions ammoniacal, then slightly acid with acetic acid. Any iron present was eliminated as phosphate by adding a small quantity of disodiumphosphate (to insure a sufficiency of phosphoric acid) and filtering. In the filtrate acidified by acetic acid the calcium was precipitated by ammonium oxalate, filtered off and weighed as oxide. The filtrate was made ammonical and after standing the magnesium precipitate was collected by filtration and weighed as pyrophosphate. The results of these determinations of the calcium and magnesium in the sera and corpuscles were as follows:

1. 40 c.c. normal goat serum contained CaO 0.0054 gr., MgO 0.00082 gr.
2. 40 c.c. immune " " " " 0.0064 gr., " 0.00243 gr.
3. 40 c.c. immune goat serum, treated with washed sheep's corpuscles, centrifuged and drawn off clear " " 0.00625 gr., " 0.00225 gr.
4. Corpuscles from 100 c.c. blood which had been exposed to heated immunized goat serm " " 0.00307 gr., " 0.00182 gr.
5. Washed corpuscles from 100 c.c. sheep's blood " " 0.00255 gr., " 0.00212 gr.

Control determinations of the calcium and magnesium in (4) and (5) by the more exact method of Chizynski gave approximately the same results as above. The assay of such minute amounts of these substances is necessarily attended by proportionally large errors. However, the results obtained for the amounts of CaO and MgO contained in immunized serum before and after mixture with corpuscles and that contained in sensitized and non-sensitized corpuscles correspond nearly enough to make it quite certain that there is no interchange of these substances of the kind suspected. Of interest is the fact that the analyses

show a smaller amount of MgO in normal serum than in immunized serum. Whether this relation holds good between all normal and immunized animals as well as between the two from which the sera used in the above determinations were obtained would be important to ascertain. This will be looked into further as soon as other immunized animals are available.

Although a relation of magnesium soaps to the complex hemolysins of the blood has not been established by these experiments, their existence in blood serum and their hemolytic properties are quite a certainty. A confirmation of the observation of Hahn that by the action of an enzyme fat is formed in normal blood but not in immunized blood *in vitro* would be a positive indication of some relation of the hemolysin either to fats, derivatives of fats, their progenitors or the fat-forming agent. As already stated, the fatty acids in the alcohol extracts from blood are probably products of the manipulation, and though one might even suspect the magnesium soap to be of the same origin, the possibility of establishing a relation between fat substances in blood and complex hemolysins makes this inquiry one which merits further pursuit.

In conclusion I wish to thank Dr. L. Hektoen, who suggested this work, for many valuable hints and constant courtesy.